THE DETERIORATION OF RAW AND REFINED SUGAR CRYSTALS IN BULK.

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Through the kindness of Messrs. Steel and Walton, of the Colonial Sugar Refining Co., I was furnished with several samples of cane sugar which were undergoing a slow process of inversion, that is, the cane sugar was being altered into invert sugar The samples included various grades of the best class of refined sugars, and to all appearance consisted entirely of saccharose.

The first sample was taken from a laboratory sample bottle which had been opened many times to test the progress of the inversion. It was a small-grained moist refined sugar, and during storage in a loosely corked bottle, had shown the following percentages of reducing sugar. For the figures I am indebted to Mr. Walton.

Date.	Storage.	Reducing Sugar %.
12-3-01	0 days	0·21
19-4-01	38 days	0·61
25-5-01	74 days	0·83
25-6-01	105 days	1·01

The bacteriological examination of two portions gave the following relative numbers of microscopic fungi.

	i.		ii.	
Bac. levaniformans, normal type ...	4	5	2	7
,, derived type ...	1		5	
Inert bacteria	4		—	
Streptothrix sp.	7		4	
Aspergillus glaucus	5		6	
	21		17	

With larger quantities of the sample, greater numbers of colonies were obtained, but they did not differ in any great degree,

nor did they include any other active organism than in the tests tabulated above. The method employed for separating the bacteria was the ordinary one of infecting molten agar with a few crystals of the sugar and pouring the agar into a Petri dish after the sugar had dissolved. From several plates thus prepared, one containing from 15 to 30 colonies was taken, and every colony was infected into nutrient agar and into saccharose peptone, prepared as described in a preceding paper (*antea*, p. 592). When in about three or four days, the saccharose peptone in some of the tubes had become white and opaque, all the saccharose peptone cultures were tested for gum by precipitation with alcohol, and for reducing sugar with Fehling's solution. The active bacteria were thus indicated, and the further identification of the bacteria was made from the agar culture.

With regard to the bacillus, both were types of the organism already described as the gum bacillus, *Bac. levaniformans*, which has been shown to rapidly cause the inversion of saccharose in solution. The inert bacteria had no action upon sugar, and were not investigated further. The streptothrix slowly inverted saccharose. The aspergillus was a race of *Aspergillus glaucus*, which readily formed aerial ascospores. The inverting action of *Aspergillus* is well known.

The second sample was a soft-grained refined but not quite white sugar. The colonies that developed on the infected agar plates were found to consist of the gum bacillus alone. One portion contained the derived type, another contained a majority of the derived type, with a few colonies of the normal bacillus.

The third sample was similar to the second, but of a slightly darker shade. Compared with the second sample, it was nearly sterile, but the few colonies that developed consisted of the derived and the normal type of *Bac. levaniformans* in the ratio of about 1 to 6.

A fourth sample of white crystalline refined sugar contained the normal bacillus; only one colony of the derived type was obtained from one of the portions.

A sample of raw sugar solution of the consistency and appearance of thin molasses was, when received, slowly fermenting, that

is to say, bubbles of gas were being given off. A film of the solution showed the presence of a yeast, and the gum bacillus of the normal type was isolated by plate culture.

A moist raw sugar had been tested in the laboratory of the Colonial Sugar Refining Co., and had shown the following percentages of reducing sugar :—

10-5-01	1·43
25-5-01	2·90
25-6-01	4·46

From an agar plate infected with the sample, there were obtained 30 colonies of the normal type, 3 of the derived type, and 1 inert bacterium.

A soft yellow refined sugar which contained 2·5 % reducing sugar, and which had undergone no change during a storage period of $3\frac{1}{2}$ months, contained, for every 12 inert bacteria, 2 of the normal and 1 of the derived type.

Another similar sugar which contained the same percentage of reducing sugar, and which had shown no further inversion in three months, contained, for every 30 inert bacteria, 15 bacilli of normal type.

A raw sugar which had been damaged by water in November, 1895, showed a very heavy inversion. The water content, after the damage had been done, was 6·97 %. The analyses which were made in the Sugar Company's laboratory from time to time, are as follows.

Date of Analysis.	Reducing Sugar %.
Nov. 22, 1895	0·9
,, 25, ,, ...	3·1
,, 27, ,, ...	4·3
Dec. 11, ,, ...	9·6
,, 24, ,, ...	11·6
Feb. 20, 1896	15·6
Nov. 1, 1898	20·8
June 20, 1901	26·3
Nov. 13, 1901	32·7 { dextrose 18·9 / levulose 13·8

The bacteriological examination of this interesting sample showed that it contained the bacilli of the normal type in practically pure culture, there being one inert bacterium to every ninety-nine active bacilli.

It is apparent from the bacteriological examination of these samples that the inversion is the direct result of the growth of the bacillus which I have described in the previous paper and to which the name of *Bac. levaniformans* has been given. Indeed it was from these samples that the numerous races of the bacillus were obtained. The organism has been already shown to be capable of growing in solutions of cane sugar containing but a trace of nitrogenous food, as for example in solutions containing one-thousandth of a per cent. of peptone. When growing in this poor medium it alters the sugar so much as to produce a visible formation of gum in a few days. In view of this faculty of growing in poor media and of the fact that an inversion of sugar accompanies the growth, there can be no doubt that it is alone responsible for the inversion of the crystals in bulk, and that the chief condition for its growth is a more or less moist state of the sugar and a warm temperature.

It has been already noted that the relative formation of gum levan is less and the inversion of sugar greater in poor nitrogenous than in more nitrogenous solutions. In refined sugar crystals the amount of nitrogenous matter is infinitesimal, and it may be that the gum-forming faculty is entirely in abeyance, since no gum is found in such sugars.*

But although this hypothesis may partially explain the absence of gum in bulk sugar, there is another property of the gum cultures that must be taken into account. During the prolonged cultivation of the bacillus, the solutions of sugar which during the height of the fermentation are white and opaque, gradually, as time goes on, become more and more translucent. The gum

* For example, the sample which had heavily inverted and which contained 26·3 % reducing sugar had only 0·19 % of gum and insoluble organic matter precipitable by alcohol.

precipitable by alcohol becomes less, and at the same time the invert sugar increases. This is proved by the analysis of a 50 days' culture which in the following table is compared with the analysis during the height of the fermentation. As in the previous paper, the figures are expressed in parts (grms.) from 100 of the original saccharose.

Gum and Reducing Sugars in Old Cultures.

	12 days.	50 days.
Mixed reducing sugars ...	62	71
Crude gum	31	22

The slow hydrolysis of the gum is probably the result of the action of the acids secreted by the organism and contained in the culture fluid. In a previous paper (*antea*, p.595) the solvent action of the acid upon the gum was noted, and it was seen that when the acidity of the culture was not neutralised the gum was incompletely precipitated, and during the process of eliminating the alcohol it was partly (or entirely) converted into reducing sugar. But to place the hydrolytic action of the acid, which is chiefly lactic, beyond doubt, the following test was made. A fragment of pure gum was dissolved in water and a portion of the solution tested with Fehling's solution. There was no reduction of the copper hydrate. Another portion was boiled for 30 seconds with an equal volume of normal lactic acid, neutralised and tested, when a copious precipitate of the red suboxide was obtained. This test places the action of the acid beyond doubt.

The action was also tested quantitatively. The acidity of the 50 days' culture was first tested and found to be equal to 0·9 c.c. normal lactic acid in every 100 c.c. of culture fluid. Then 1·388 grms. of gum, free from reducing sugars and dried at 100° C. for several days, were dissolved in 100 c.c. of water to which 0·9 c.c. normal lactic acid was added. The solution was heated at 80° C. for three hours, and after being cooled was made up to volume. The sugar was estimated and found to be equal to 1·21 grms. of reducing sugar. Assuming the gum to have been 96 % pure and to have the formula $C_6H_{10}O_5$, the determination showed that

80% was converted into sugar by heating for three hours at 80° C. with an amount of lactic acid equal to that found in the cultures.

The deterioration of Hawaiian raw cane sugar was investigated by Shorey* about three years ago. In the paper he mentions that one of the reasons put forward to account for the inversion of the sugar is a fermentation caused by bacteria, prominent among which are those producing lactic and butyric acids. At another place he writes :—

"It is generally accepted that the butyric ferments are without the power of inverting cane sugar, while the lactic ferments sometimes seem to have this power. It seemed to me, however, very unlikely that the inversion could be brought about by bacteria. The sugar was quite dry, the crystals separate and distinct, and in appearance was like so much air-dried sand. Even in December, when the deterioration had reached 4·0°, although the sugar was moist, each crystal was still separate and distinct, being simply coated with a thin syrupy film. Bacteria can only reach development in a liquid or semi-liquid continuous medium, and sugar, so long as it continued so dry that the crystals remained distinct and separated by air spaces, would necessarily prove a medium ill-adapted for the growth of such bacteria as produce lactic or butyric acid. Moreover, all the sugars examined showed as the result of deterioration a very small amount of acid and a comparatively large amount of invert sugar. Now if lactic acid ferments were the invertive agents, the processes of inversion and production of acid would undoubtedly go on together or so closely that they would seem simultaneous. I was led to conclude then that the inversion was not produced by lactic or butyric ferments, but by some other agent."

Shorey did not search for bacteria, but he discovered the mycelia of *Penicillium glaucum* distributed among the crystals, and concluded that this ubiquitous mould was the active inverting agent in the particular samples.

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* Shorey, Journ. Soc. Chem. Industry, xvii., 555.

With regard to the inverting action of the ordinary lactic bacteria, it may be said that any invertive power which they have must result from the hydrolytic activity of lactic acid, for according to Reynolds Green, and also Lafar, there are but few bacteria known which secrete invertase, and these do not include the lactic bacteria. Lactic acid undoubtedly causes the inversion of saccharose, and there is no reason why a comparatively small amount of acid should not in an indefinite time produce a comparatively large inversion. I have quoted Shorey chiefly to show how one might be led astray by *a priori* reasoning, for his remarks about the Hawaiian sugars would apply equally to these Australian samples. *Bacillus levaniformans* is an organism that plays many parts. It is at once a lactic and a butyric acid ferment, and it is capable of inverting a comparatively large amount of sugar, probably through the action of invertase, for the heavy inversion points to the presence of this enzyme.* Furthermore, it cannot be doubted that its spore-forming faculty and its gum capsule enable it to survive and vegetate under conditions which would be adverse to most other forms of bacterial life.

And now a word about the distribution of the organism. The habitat of *Bac. levaniformans* is not restricted to one set of mills and one refinery, but it is widely distributed. It may be the cause of the deterioration of the Hawaiian sugars, and it certainly occurs in the Australian mills. I have examined a set of samples of raw and refined sugars from various parts of the world. These I received from the Colonial Sugar Refining Co., with the information that the sugars had been kept in tightly stoppered bottles and had been opened only to send me smaller samples. The

* A sterile culture solution with 0·08% lactic acid (= the acidity of a culture) was placed in the incubator for four days at 37°. At the end of this time 2·8% reducing sugars were found. As the acidity in a culture of the bacillus does not reach 0·08% for 24 hours, we should allow a day and compare the four days' inversion by acid with a five days' inversion by bacteria. The great difference in the amount of reducing sugars, between 2·8 (a four days' acid inversion) and 50 (the reducing sugars in a five days' culture) shows that a strong inverting agent is secreted by the bacilli.

result of the investigation was as follows. The qualification—few, many, or very many—indicates in a rough manner the number of colonies obtained from about 1 grm. of sample.

Source and quality of sugars.	*Bac. levaniformans.*
1. Demerara, ordinary pale	absent.
2. ,, ordinary yellow	few present.
3. Mauritius, ordinary white	many present.
4. Peruvian, good quality crystal	very many present.
5. Egyptian, good quality raw sugar	many present.
6. ,, ,, ,, ,, ,,	many present.
7. Java, ordinary good	very many present.
8. ,, stroops, very low moist raw sugar	many present.
9. German, low beet sugar	very many present.
10. ,, ,, ,, ,,	many present.
11. ,, granulated refined	sterile.
12. Russian, refined beet	few present.
13. French ,, ,,	many present.
14. Fiji, raw crystals	very many present.

Since the organism is contained in sugars from such distant places, it is safe to say that its distribution is universal.

Perhaps it would not be out of place to indicate how the sugar technicist should test for the organism. In a litre of tap water there are dissolved 100 grms. saccharose, 1 grm. peptone, 5 grms. potassium chloride, and 2 grms. common sodium phosphate. The solution is filtered, if necessary, and about 100 c.c. are filled into clear glass bottles, which are plugged with cotton wool. The bottles are placed in a steamer (a potato steamer would do) and steamed for twenty minutes on three successive days. They are then kept as near 37° C. as possible for 10-12 hours and again steamed. This is followed by keeping at 37° for two days, and those bottles which remain bright are sterile. All this procedure is necessary, because of the possible presence of *Bac. levaniformans* in the sugar. Into the sterile solutions about two grms. of the suspected sample are introduced by means of a small spoon or spatula, which has just cooled after having been sterilised by

heating in a flame. The infected bottles are maintained at 37°. In from two to four days the presence of *Bac. levaniformans* is shown by the fluid becoming milky. A turbidity of the solution must not be confounded with the opaque milkiness which is characteristic of *levaniformans*. At the same time it must be borne in mind that some races produce but a faint opalescence that might easily be mistaken for a turbidity of the solution. In all cases it is advisable to extract a small loop or a tiny drop by means of a sterile looped platinum wire, or a sterile dropping pipette, and infect a fresh bottle. In from 5 to 7 days, when the formation of levan is at a maximum, the second culture could be tested for reducing sugars and also for levan by precipitation with alcohol and subsequent solution in water.

Of course, the bacteriologist would also test the sugar straight away by preparing plates from saccharose-agar or even ordinary nutrient agar, infected with about 1 grm. of the sample. Any suspicious colony that developed would be picked out, infected into the saccharose fluid medium, and from this, as a starting point, he would proceed to identify the organism. The primary plate method is much more satisfactory than fluid cultures, because foreign organisms, in some cases, appear to hinder the development of *Bac. levaniformans*.

So far as the economic importance is concerned, it is, perhaps, impossible to estimate the loss entailed directly or indirectly through the activity of the organism. In many cases raw sugar has been known to have deteriorated during storage at the mill, in transit to and in storage at the refinery. At one refinery in Sydney the loss through inversion during the storage of the raw sugar has at times been considerable. During the last three years, however, the Colonial Sugar Refining Company, suspecting the loss to have been due to the activity of micro-organisms, have altered their methods of manufacture, and the sugar is now less subject to change. In three years the average loss through deterioration has been reduced from 0·5 % to 0·1 % on the whole stocks. This means that the loss of pure sugar on an annual stock of say 50,000 tons, which three years ago was 250 tons,

has now been reduced to 50 tons. And as the sugar destroyed is transformed into objectionable substances, the removal of which in the process of refining entails a further loss of a similar quantity of pure sugar, there is thus a double loss, the money value of which was about £5,000 three years ago, while now it is £1,000. I have these figures from Mr. T. U. Walton, the chief chemist to the Colonial Sugar Refining Company. The degradation might have been caused by many agents, but one is probably justified in ascribing over 95 % of it to the action of *Bac. levaniformans*.